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ABSTRACT
Alterations in sialylation are well documented in breast cancer as well as other epithelial cancers. A principal enzyme implicated in this process is the sialyltransferase ST6Gal, which mediates the synthesis of the sialyl a2,6-anomeric linkage to terminal lactosamine structures on glycoproteins, and high levels have been linked to a poor prognosis especially among patients with locally advanced stage III breast cancer. Transcription of the ST6Gal gene is regulated by selective use of multiple promoters in a tissue- and developmental-specific manner. We documented the existence of a breast-specific promoter that is recruited de novo to allow high ST6Gal gene expression during late pregnancy and lactation in mouse mammary glands. However we failed to detect an equivalent breast-specific promoter operative in humans. Examination in a number of murine breast cancer models also failed to detect recruitment of this promoter in ST6Gal expression.

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Table of Contents

INTRODUCTION

Alterations in sialylation are well documented in breast cancer as well as other epithelial cancers. In particular, elevated cell surface sialylation may mask tumor cells from immune surveillance. Of equally importance to the long-term survival of breast cancer patients, increased cell surface sialylation may decrease intercellular adhesion, directly contributing to the ability of tumor cells to metastasize. A principal enzyme implicated in this process is the sialyltransferase ST6Gal, which mediates the addition of sialic acid via an α2,6-anomeric linkage to terminal lactosamine structures on glycoproteins. High levels of ST6Gal have been linked to a poor prognosis especially among patients with locally advanced stage III breast cancer [1,2]. In normal individuals, serum and mammary gland ST6Gal levels are also elevated during late pregnancy and lactation. Among all animal species examined, including human, bovine, rat, and mouse, transcription of the ST6Gal gene is regulated by the selective use of multiple promoters in a tissue- and developmental-specific manner.

BODY

This work was initiated by our original finding that a novel ST6Gal mRNA form is expressed in the lactating mammary glands of the mouse. This prompted our hypothesis that the novel mRNA form originates from a heretofore unique ST6Gal promoter. Further, we reasoned that a human homologue of this "lactation-specific" may exist and that it's inappropriate utilization may generate the elevated ST6Gal expression during breast cancer.

The key research accomplishments to this concept award are as follows:

1. We have successfully characterized the murine "lactation-specific" ST6Gal mRNA. We conclusively demonstrated that it originates from a unique promoter/transcription regulatory region that is expressed only in mammary glands and only during late pregnancy and lactation. We have mapped this promoter region on the murine ST6Gal gene, *Siat1*. These findings have been published [3].

2. We established that elevated ST6Gal mRNA during lactation is not due to contribution of lymphocytes during this process since an identical increase was also observed in lactating mammary glands of SCID mice.

3. We examined the expression of ST6Gal in a number of mouse breast cancer models, including mouse breast carcinoma lines 410.4, SHI, N2O2-neu, and BF-C3. Further we also experimentally induced mouse mammary tumors using polyomavirus middle-T antigen. In none of these cases were we able to observe utilization of this novel ST6Gal promoter.

4. We examine mRNA derived from normal and lactating human breasts. After exhaustive search, using RT-PCR and 5'-RACE, we failed to detect an equivalent human form of the murine lactogenic ST6Gal mRNA form.

CONCLUSION

Our findings are not consistent with the utilization of a unique promoter in the over-expression of ST6Gal during breast cancer. So far, we have no evidence for the human equivalent of the novel transcription promoter documented in the mouse.

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APPENDICES

1 reprint: Dalziel et al, Glycobiology 11, 407-412 (2001).

Mouse ST6Gal sialyltransferase gene expression during mammary gland lactation

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The sialyltransferase ST6Gal mediates the biosynthetic addition of sialic acid, via an α2,6 linkage, to the nonreducing end of terminal lactosamine structures. Transcription of the murine ST6Gal gene, Siat1, is regulated by the selective use of multiple promoters in a tissue- and development-specific manner. Here we report that Siat1 mRNA expression is dramatically elevated in lactating (relative to virgin) mouse mammary gland. The predominant ST6Gal mRNA species expressed in lactating mammary gland is a heretofore undocumented isoform containing a unique 5'-untranslated region originating from the mouse Siat1 genetic region, now defined as Exon L, residing 549-bp 5' of the previously characterized Exon X₂. Thus, the novel ST6Gal mRNA form initiates transcription from the region designated as p4 and incorporates the unique sequence from Exon L in 5'-juxtaposition to commonly shared sequences encoded on Exon I to Exon VI. In contrast, cells derived from virgin mammary tissue expressed only the housekeeping mRNA form derived from p3, with Exon O sequence preceding Exons I–VI. The Exon L–containing, p4 class of mRNA was also not detected in a survey of eight other mouse tissues.

Previous reports have indicated a strong correlation between mammary cancers and elevated ST6Gal expression in rats and in human patients. However, we uncovered neither elevated expression of ST6Gal mRNA nor appearance of p4 class in mouse breast carcinomas experimentally induced by transformation with the polyoma-middle T oncogene. A number of established breast carcinoma cell lines were also examined, with ST6Gal mRNA and activity generally low. Moreover, with the exception of the Shionogi cell line, p4 class of ST6Gal mRNA was not expressed in any of the mouse breast carcinoma specimens examined.

Taken together, our data indicate that murine ST6Gal induction during lactation is achieved by *de novo* recruitment of a normally silent promoter. Furthermore, the data provide no support for elevated *Siat1* expression on the mRNA level in association with murine mammary gland carcinogenesis. With the single exception of the Shionogi cell line, the p3 class remains the predominant ST6Gal mRNA expressed in all other murine mammary carcinoma cells examined.

Key words: lactation/mammary gland/mouse/sialyltransferase/ ST6Gal

Introduction

The sialyltransferase ST6Gal (CMP-Neu5Ac:Galβ1,4GlcNAc-α2,6-sialyltransferase) (Tsuji *et al.*, 1996) is responsible for the biosynthetic addition of sialic acid (Sia) from CMP-Sia to the lactosamine termini (Galβ1,4GlcNAc) of glycoproteins in an α2,6 anomeric linkage. Significant homology exists between cloned human, bovine, rat, and mouse genes coding for ST6Gal (Mercier *et al.*, 1999). In mouse, this gene, *Siat1*, is located on chromosome 16 (Kalcheva *et al.*, 1997) and is the only gene encoding a glycosyltransferase that synthesizes the SA(α2,6) to Galβ1,4GlcNAc-R structure (Hennet *et al.*, 1998). The *Siat1* encodes an open reading frame spanning five separate exons, and, in addition, a complex array of 5' untranslated exons exist upstream of these coding regions. These 5'UT exons are utilized in a tissue-specific manner as the result of usage of multiple promoter regions (Hu *et al.*, 1997; Wuensch *et al.*, 2000).

During lactation, sialyltransferase enzymatic activity is significantly elevated in the serum of rat (Bushway *et al.*, 1979) and bovine (Sherblom *et al.*, 1986) but not human (Rajan *et al.*, 1983). In rat, high sialyltransferase activity in the mammary gland during proliferation and involution accompanies the high activity found in serum (Ip, 1980). Elevated serum and tumor sialyltransferase activity has been reported in human breast carcinoma (Abecassis *et al.*, 1984; Dao *et al.*, 1986), and high ST6Gal levels have been linked to a poor prognosis (Recchi *et al.*, 1998b). Elevated serum and tumor sialyltransferase activity has also been experimentally reproduced in rat breast carcinomas (Fox *et al.*, 1981).

We report here that ST6Gal is dramatically induced in the mouse mammary gland during lactation. This ST6Gal mRNA induction is mediated by recruitment of a novel 5'UT exon, exon L, probably driven by a lactogenic promoter, tentatively named P4.
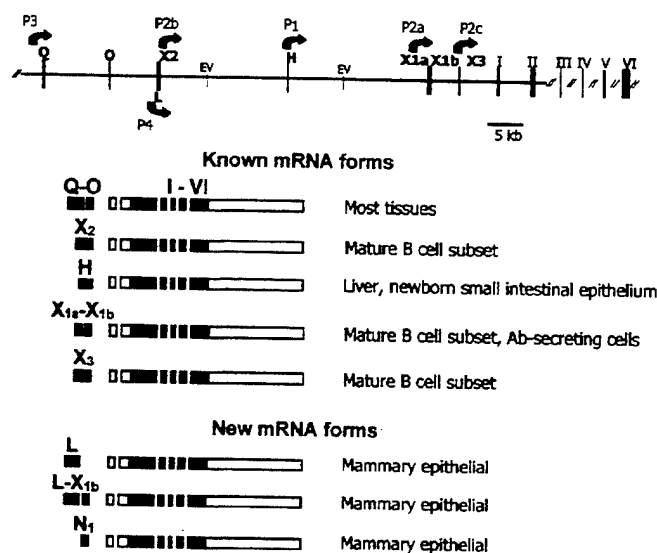
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[1]To whom correspondence should be addressed

Results

A novel ST6Gal mRNA isoform is expressed in mammary gland during lactation.

Members of the ST6Gal mRNA family differ only in their 5′-UT domains but share identical downstream sequences (including the protein coding domain) encoded on Exons I to VI (see Figure 1). A 5′-RACE (rapid amplication of CDNA ends) strategy was devised to qualitatively assess differential expression of the ST6Gal mRNA family. RNA from lactating mammary gland of a day 9 postpartum FVB × (FVB(C57 × CBA)) mouse (sample LM) was reverse-transcribed using mST1-P1, an anti-sense primer complementary to a region in the shared Exon II. The reverse-transcribed 5′ ends to ST6Gal mRNAs were polymerase chain reaction (PCR) amplified using a nested primer, mSTI-md11, complementary to a region in Exon I. The major product, visualized by ethidium bromide staining, migrated at around 300 bp (Figure 2, bottom, lane 1), consistent with a divergent region 5′ of Exon I–encoded domain.

Eighteen clones representing the md11-derived PCR product were selected at random and sequence analyzed. All 18 clones were derived from ST6Gal mRNA as evidenced by the presence of Exon I sequence. The majority of these clones (15) contain a novel sequence immediately 5′ of Exon I. This novel 5′-UT sequence originates from the mouse *Siat1* genetic region, now defined as Exon L, located only 549 bp 5′ of the previously charaterized Exon X_2 (Wuensch *et al.*, 2000). Exon

```
tgtccatttt  ttttccctgc  ttcatgactg  tcacctatag  agcagtgttt
tagtcagggt  ttttattcct  gcacaaacat  catgaccaag  aagcaagttg
gggaggcaag  ggtttatttg  gcttacactt  ccatactgct  gttcatcacc
aaaggaagcc  aggactggaa  ctcaagcagg  tcaggaagca  ggagctgatg
cagaggccat  ggagggatgt  tctttactgg  cttgcttccc  cgggcttgct
cagcctgctc  ttttatagaa  ccaagaatac  cagcccagag  atggtcccac
ccacaagggg  cctttccccc  ttgatcacta  attgagaaaa  tgccttacag
ttggatctca  tggaggcatt  tcctcaactg  aagctcnttt  ctctgtgata
actccagctg  tgccaagttg  acacaaaacc  agccagtaca  agcaggtaga
ggctttgtgt  ttcgcagtcc  ctngaagtga  gctcagttac  tggccctgca
gtcatttggt  gctcccacat  ctgtaaatag  ctcgaggctt  tggcaagcct
gggtgcatga  tctntggagg  aaggagatgg  ggtgagctga  gtacagtgct
cagatttgac  aggtcctgtg  aagtcagggc  acctgactgg  ggtagggaaa
tgaaggccag  cgGCCAGGGA  GCAATGCGAG  AGTGTAAGGG  ACTATGGGTT
CTAAGGACGG  TACAGCTTGG  CGGGTGGGGA  GGAAAGCGTG  TACTGCTCTT
GCCTGCTAAG  CTGAGAAATT  TCAACAGGGC  AGATTAAACA  TAGAGACTGT
                                                        ◄━━━
TTTAAGCGTC  TGAGGCACTT  TTGATTCTTC  AGAGCTAGTT  TTGAGTGAGA
◄━━━━━━━━━●mSTI-LP1            ●mST1-LP2
CTTCATCTGG  TACAGgtaag  gcatttgatg  cagaggttgg  ttaggaggct
gtggtgaggg  tcttgaaggt  aggtatggtt  ctggttcttc  ttcccttctt
gggagtctgg  tgctctggca  caccagtttg  tagaagacag  ggccaagcat
```


- 300 bp
- 200 bp
- 100 bp

1   **2**   **3**
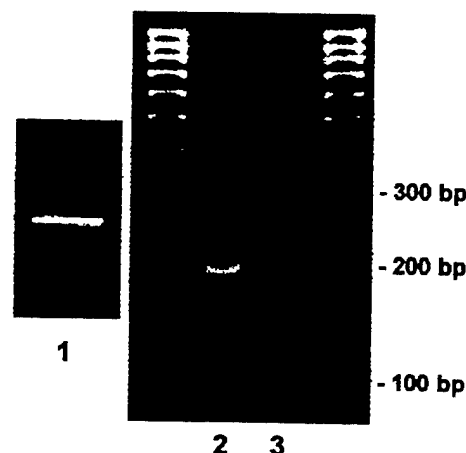
Fig. 2. Determination of transcription initiation of Exon L–containing mRNA form. The genomic sequence flanking the putative transcription initiation site for Exon L–containing ST6Gal mRNA form is shown. Sequence shown in capital letters denote the transcribed Exon L region; sequences in lowercase letters are the flanking 5′- and 3′-regions. The predicted transcription initiation point is the junction between the 5′-flanking sequence and the Exon L sequence. The EtBr visualized gel illustrated on the bottom is the 5′RACE and primer extension analysis of the Exon L–mRNA form. Lane 1 is the RACE analysis of *Siat1* mRNA from day 9 lactating mammary gland (LM) using primers md11 and AP1 (see *Materials and methods*), generating predominately a 300-bp product. The RACE product was sequence-analyzed after cloning into pCR22.1 and confirmed the dominant presence of Exon L as the *Siat1* mRNA 5′ leader region (see Table I). Lanes 2 and 3 are primer extension/5′RACE products using primers mST1-LP-1 and mST1-LP-2 on the LM sample (see *Materials and methods*). The products from primer extensions with primers mST1-LP-1 or mST1-LP-2 are 200 bp and 215 bp ± 5bp, are shown in lanes 2 and 3, respectively, and are consistent with the predicted transcription initiation point as shown in the genomic sequence above. The annealing sites for primers mST1-LP-1 and mST1-LP-2 are as shown.

X_2 contributes to a another 5′-UT motif in a B cell–specific ST6Gal mRNA species (see Figure 1). Exon L resides 48 kb 5′ of Exon I and 18 kb 5′ of the liver-specific Exon H. Among the three remaining RACE clones that do not contain Exon L, one contained the Exon O sequence found in the constitutively

Known mRNA forms

Q-O   I - VI
Most tissues

X_2
Mature B cell subset

H
Liver, newborn small intestinal epithelium

X_{1a}-X_{1b}
Mature B cell subset, Ab-secreting cells

X_3
Mature B cell subset

New mRNA forms

L
Mammary epithelial

L-X_{1b}
Mammary epithelial

N_1
Mammary epithelial

Fig. 1. Genomic organization of the mouse ST6Gal gene, *Siat1*, and summary of known ST6Gal mRNA forms. The top panel schematically represents the genomic organization of the mouse ST6Gal gene, *Siat1*. Exon sequences are denoted by vertical bands. Invariant exons shared among all ST6Gal mRNA forms are labeled in roman numerals (I–VI); the remainder of the exons (Q, O, L, H, X1a, X1b, X2, X3) specify the divergent 5′-untranslated regions of the ST6Gal mRNA family. Curved arrows indicate the transcription initiation points and putative promoters regulating the differential expression of the ST6Gal mRNA forms. A summary of the known ST6Gal mRNA forms is shown in the middle panel. The lower panel summarizes the structure of the novel mRNA forms reported here.

expressed P3-mRNA form. Another clone specified an Exon X_{1b} sequence interrupting between Exon L and Exon I sequences. The last RACE clone contained another novel sequence of 86 nts 5' of Exon I, tentatively termed N_1. At present, the origin of the N_1 sequence is not known.

Three other RNA samples of mouse mammary epithelial origin were also subjected to the identical 5'-RACE procedure; the results are summarized in Table I. One of them, an immortalized cell line derived from normal mouse mammary gland (NMG), yielded 17 RACE clones. In striking contrast to RACE clones derived from lactating mammary gland, there were no Exon L forms among these 17 clones. Instead, the vast majority (12) were Exon O–containing P3 forms. Among the remaining five clones, four represented N_1 forms, and one represented the Exon H–containing P1 (hepatic) form. Another RNA sample was from the mouse breast carcinoma line 410.4, from which 14 RACE clones were generated and analyzed. The 5'-RACE profile of 410.4 was similar to that of cells derived from normal mammary epithelium; 12 410.4 clones were Exon O–containing P3 forms and the remaining two were Exon L forms. However, the Siat1 mRNA profile of Shionogi (SHI), another mouse breast carcinoma cell line, was similar to that of lactating mammary gland. Eight SHI-derived RACE clones were sequence analyzed; all eight clones represented the Exon L form.

To more precisely define the 5' end of this Exon L–containing ST6Gal mRNA species, primer extension coupled with PCR amplification (5'-RACE) was performed using new primers that would require only minimal extension by the reverse transcriptase. For this purpose, mST1-LP-1 and mST1-LP-2, both complementary to Exon L, were designed. A 200 (± 5)-bp product resulted when mST1-LP-1 was used as specific primer for both the reverse transcription and PCR steps. In contrast, a 215 (± 5)-bp product results when mST1-LP-2 was used. Taking into account the contribution of the synthetic 5' universal primer (45 bp), both mST1-LP-1 and mST1-LP-2 predict a 200 (± 5)-bp Exon L. The transcription initiation of the Exon L–mRNA species as shown in Figure 2 (top) specifies a 203-bp Exon L predicted from the longest 5'-RACE product that was sequence analyzed.

P4-form of ST6Gal mRNA is restricted to mammary glands during lactation

The data shown up to this point was derived from mouse strain FVB × [FVB(C57×CBA)]. To confirm that the novel P4 (Exon L–containing) mRNA form is expressed during lactation in a different mouse strain, the mouse strain CBA was examined. Mammary gland RNA was isolated from virgin, day 14 preg-

nancy and day 5 postpartum CBA animals. As assessed by a probe against the shared Exon II, there was a striking increase (over fivefold) in overall ST6Gal mRNA levels postpartum (Figure 3A). Mammary gland from day 14 pregnant animals, on the other hand, showed only a modest increase (around twofold) when compared to mammary gland from a virgin animal. When subjected to 5'-RACE analysis, virgin mammary gland expressed only P3 form, commonly regarded as the constitutively expressed ST6Gal mRNA form, and only P4-type (Exon L–containing) clones were recovered from mammary gland of day 5 postpartum animals. A mixture of P3 and P4 clones was recovered from 5'-RACE analysis of mammary glands from animals in the 14th day of pregnancy (Figure 3C).

Mammary gland during lactation expresses high levels of ST6Gal mRNA, the bulk of which are from de novo appearance of the P4-mRNA form. The level of ST6Gal mRNA during lactation is higher than that seen with resting liver and around two thirds of that seen in turpentine mediated acute phase liver (Dalziel et al., 1999).

A Northern blot panel of mouse tissues was used to assess the range of P4-mRNA expression (Figure 4). The level of overall ST6Gal mRNA expression, as measured by a probe against the shared Exon II, varied among tissues examined. The P4 form, as visualized by a probe against Exon L, was present only in lactating mammary gland (Figure 4). In contrast, the constitutively expressed P3 form, as visualized by

C	Virgin	D-14	D-5PP
Total	7	27	12
O - I	6	8	-
L - I	-	15	12
N_1 - I	1	2	-
unidentified	-	2	-
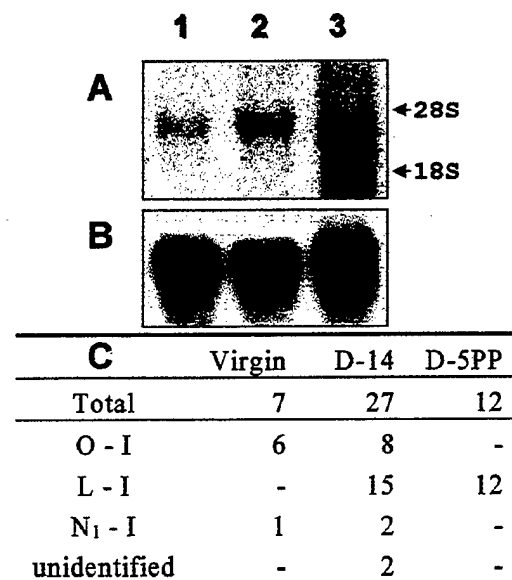
Fig. 3. Regulated expression of *Siat1* in mammary gland during pregnancy and lactation. (A) A northern blot profile of ST6Gal mRNA expression in mammary glands of virgin (lane 1), day 14 pregnant (lane 2), and day 5 postpartum (lane 3) strain CBA mice. Thirty micrograms of RNA is analyzed in each lane; the blot was hybridized using *Siat1* 0.75 kb Exon II probe, which visualizes all ST6Gal mRNA forms. Migration points of the 28S and 18S rRNA bands are indicated. (B) is the same blot stripped and rehybridized with a mouse 18s cDNA probe to control for equal loading of the lanes. The same panel of mammary gland RNA was subjected to 5'RACE using mST1-MD11 and AP1, the products of which were cloned and subjected to sequence analysis as described in *Materials and methods*. (C) summarizes the resultant 5'RACE clones based on sequence distribution of the region in 5'-juxtaposition to Exon I sequence.

Table I. Composition of ST6Gal cDNA from 5'-RACE

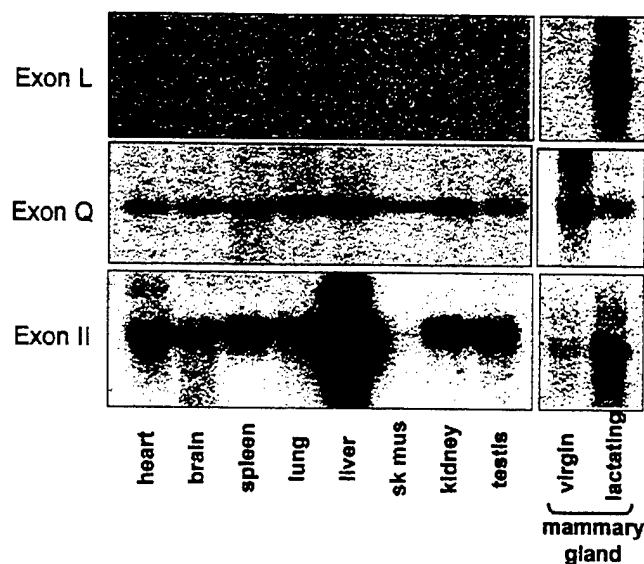| Type	LM	NMG	SHI	410.4
Total clones	18	17	8	14
O–I	1	12	0	12
L–I	15	0	8	2
L–x_{1b}–I	1	0	0	0
H–I	0	1	0	0
N_1–I	1	4	0	0

Fig. 4. Tissue specificity of Exon L–containing ST6Gal mRNA forms. Multiple mouse tissue mRNA blots (Clonetech Laboratories) were probed for sequences unique to (from top to bottom panels) Exon L, Exon Q, or Exon II. The right panels were parallel analysis of RNA from virgin and lactating mammary glands. Differential probes for Exons L, Q, and Exon II were synthesized against PCR-generated fragments of the respective exon sequences and labeled with ^{32}[P]-dATP or ^{32}[P]-dCTP.

a probe against Exon Q, was present in all tissues surveyed, including virgin mammary gland (Figure 4).

ST6Gal expression in mouse breast cancer

Table II summarizes a survey of ST6Gal expression in a number of mouse cell lines. Lactating mammary gland (LM) and the human hepatoma cell line, HepG2, were included as references. The normal mammary epithelial line, NMG, and the mouse breast carcinoma lines 410.4, SHI, N202-neu, and BF-C3 were examined. All mammary-derived cell lines except N202-neu expressed low levels of ST6Gal mRNA, as assessed by a probe for Exon II sequences. The measured ST6Gal enzymatic activity, in general, was commensurate with ST6Gal mRNA levels. The only deviation was NMG, exhibiting a higher enzymatic activity with only 28% of the mRNA level measured in N202-neu. It was also significant that none of the cell lines examined except for one (SHI) expressed the novel P4-mRNA form. Though the overall level of ST6Gal expression was unremarkable in SHI, the P4-mRNA form was the major constituent in SHI ST6Gal mRNA pool (see Table I).

To further explore the relationship between ST6Gal expression and mammary tumorigenesis, mouse mammary tumors were experimentally induced using polyomavirus middle T-antigen. Six separate tumors were harvested and analyzed on Northern blots and by 5′-RACE for ST6Gal mRNA expression. None of the six specimens exhibited elevated ST6Gal mRNA expression when probed for Exon L or for Exon II sequences (data not shown).

Discussion

Consistent with early reports of elevated mammary gland sialyltransferase activity accompanying lactation (Bushway *et al.*, 1979; Ip, 1980), we document a dramatic elevation of ST6Gal mRNA expression in mouse mammary during late

Table II. Expression of ST6Gal mRNA and enzymatic activity in murine cells

Cell line	ST6Gal mRNA (Exon II)[a]	ST6Gal activity[b]	Major mRNA form[c]
NMG	0.25	30,900	P3
410.4	0.15	10,040	P3
SHI	0.10	4,040	P4
N202	0.90	25,800	P3
TSA-MC	0.05	2,370	P3
BFC3 (undiff)	0.10	7,860	P3
BFC3 (diff)	0.10	7,250	P3
HepG2	ND[d]	154,340	P1[e]
LM[f]	(1.00)	ND	P4

[a]Measured by northern blots probed for Exon II.
[b]Measured against asilotransferrin as exogenously supplied acceptor and expressed as dpm/h/mg protein.
[c]Assessed by 5′-RACE. P3, Exon L–containing; P4, Exon O–containing.
[d]ND, not done.
[e]Previously reported in Dalziel *et al.* 1999.
[f]LM, RNA from whole lactating mammary gland used for reference.

pregnancy and lactation. Tissue differences in ST6Gal expression can be attributed to a significant degree by differential usage of multiple and independently operating transcription initiation sites. Here we report a novel form of ST6Gal mRNA expressed only in mouse mammary gland and only during late pregnancy and lactation. *De novo* accumulation of this mRNA form accounts for the elevated level of *Siat1* expression in lactating mammary glands.

In contrast to a previous report of high levels of ST6Gal mRNA in mouse mammary gland (Takashima *et al.*, 1999), our findings indicate that only a low basal level is present in virgin mammary glands (see Figures 3 and 4). This basal level is maintained by expression of the ubiquitously expressed P3 form. The novel mRNA form is not detectable in virgin mammary gland either by Northern blot or by the more sensitive reverse transcriptase PCR analysis (data not shown). This unique mRNA form, designated as P4 to be consistent with the chronological reporting of the mouse *Siat1* forms described to date (Hu *et al.*, 1997; Wuensch *et al.*, 2000), differs by a unique 5′-untranslated region derived from an upstream exon, Exon L, in the *Siat1* locus. The data is consistent with transcription initiation of the P4-form at the 5′ juncture of Exon L (Figure 2). The expression pattern of the P4 mRNA form is consistent with that of a lactogenic-responsive gene. However, it is noteworthy that a STAT5 consensus motif of lactogenic promoters (Groner and Gouilleux, 1995; Kazansky *et al.*, 1995; Liu *et al.*, 1995) is not found within 2 kb of the predicted P4 transcription initiation site (data not shown).

Elevated ST6Gal mRNA has been reported in human breast cancer, particularly in woman with histoprognostic grade III cancer (Recchi *et al.*, 1998a), raising the intriguing possibility of aberrant recruitment of the lactogenic ST6Gal promoter in tumorigenesis. However, in a survey of a number of mammary gland carcinoma cell lines, as well as several virally induced *in vivo* tumours studied, there was no evidence for an increase in ST6Gal transcripts or for elevated enzyme activity. The rationale for this apparent discrepancy is not clear, although it should be kept in mind that this limited panel of cell lines is

unlikely to represent all normal and pathological states of mammary cells. In support of this, only one cell line, SHI, has been found to express the P4 form that predominates *in vivo* in lactating mammary glands. Moreover, the steady state level of ST6Gal mRNA in SHI is extremely low, measuring only one-tenth that observed in lactating mammary gland, and one-third to one-half that observed in the nonlactating mammary gland line, NMG.

An additional interesting observation arising from the survey of ST6Gal expression is the inconsistent correlation between ST6Gal mRNA level and the measurable enzymatic activity (see Table II). The cell line NMG, for example, exhibits enzymatic activity equivalent to that of N202-neu while maintaining a three- to fourfold lower steady-state ST6Gal mRNA level. Indeed, differential posttranscriptional regulation as a consequence of unique 5'-UTR domains in the mRNA isoforms has been proposed (Aasheim *et al.*, 1993; Dall'Olio *et al.*, 1999). However, this mechanism is unlikely to participate here because both N202-neu and NMG recruit the P3 form as the predominantly expressed ST6Gal mRNA. Considered together, the data suggest multiple mechanisms are operative in dictating ST6Gal expression in mammary glands.

The functional significance of ST6Gal elevation in the lactating mammary gland can only be speculated at this point. The obvious reason is to address demands for elevated synthesis of SAα2,6Galβ1,4GlcNAc structure, present both as free oligosaccharide in milk and also as covalent modifications in cellular and milk glycoproteins (Kobata *et al.*, 1969). Moreover, ST6Gal may also be capable of elaborating the synthesis of SAα2,6GalNAcβ1,4GlcNAc-R (Nemansky and Van den Eijnden, 1992), another structure found in abundance in colostrum (Coddeville *et al.*, 1992; Nakata *et al.*, 1993; Girardet *et al.*, 1995). Moreover, the enzyme itself may be secreted into colostrum (Paulson *et al.*, 1977) in a manner analogous to enhanced deposition of ST6Gal enzyme from liver into serum during the hepatic inflammatory response (Kaplan *et al.*, 1983; Jamieson *et al.*, 1993).

Coincident with elevated ST6Gal expression in mammary gland of lactating animals, high levels of ST6Gal are also present in the intestinal epithelium of newborn animals while nursing, and weaning is concomitant with a conversion of terminal sialylation to fucosylation (Biol *et al.*, 1991; Hamr *et al.*, 1993; Vertino-Bell *et al.*, 1994). Although the physiologic significance of these events is far from clear, a tantalizing postulate is a contribution of milk and intestinal epithelial ST6Gal to innate immunity in the newborn animals. Many of the Sia-binding pathogens exhibit a preference for the α2,3-sialyl linkage (Karlsson, 1995), but the contribution of elevated α2,6-sialyl linkage to innate immunity may be as "decoys" or "smoke screens" to foil potential pathogens (Gagneux and Varki, 1999).

Materials and methods

Materials

α^{32}P-dCTP was from ICN (UK). Mega Prime kit was from Amersham. Plasmid pCR2.1 was from Invitrogen. Trizol was from Gibco BRL. Marathon RACE kit was from Clonetech. PolyA+ isolation kit and miniprep kits were from Qiagen.

Cell lines and tissues

Cell lines NMG and SHI were obtained from central cell services, Imperial Cancer Research Fund. Cell line 410.4 was an established in-house line. All lines were grown in E4/10% fetal calf serum/pen/strep/0.5% Amphotericin B. LM was obtained from a day 9 postpartum FVB × [FVB(C57 × CBA)] mouse, sacrificed, with all four glands removed and snap frozen. CBA mammary gland tissue taken from virgin, day 14 pregnancy, and day 5 postpartum were provided by Clive Dickson, ICRF. Cell lines N202-neu (Nanni *et al.*, 2000), TSA-MC and BFC3 were kindly provided by P. L. Lollini, Department of Experimental Pathology (University of Bologna, Italy). N202-neu cells overexpress *neu* oncogene, TSA-MC are derived from spontaneous tumorigenesis in Balb/c mice, and BFC3 result from insertional mutagenesis of MMTV. The latter cell line can differentiate *in vitro* after postconfluent culture.

Mice bearing the MMTV LTR fused to the polyomavirus middle-T oncogene were generated by Guy *et al.* (1992) on a FVB background. Four heterozygous males were obtained from the Beatson Institute and crossed with Poly-T negative females (C57 × CBA) and progeny screened for Poly-T via PCR. Mammary gland tumors were isolated from Poly-T homozygotic animals and snap frozen.

RNA and enzymatic analysis

For 5'-RACE analysis unless otherwise stated, 1 μg of polyA+ RNA was annealed to the primer mST1-P1 (5'-GATGATGG-CAAACAGGAGAA-3') and reverse transcribed. MST1-P1 is complementary to a region in Exon II, such that authentic reverse-transcription events of ST6Gal mRNA must span at least the Exon I–Exon II boundary. The resultant cDNA was ligated to the marathon adaptor sequence as per instructions and subjected to PCR on a Perkin Elmer thermo-cycler, using the Touchdown program as recommended by Clonetech (Oxford, UK), using the anchor primer AP1 (5'-CCATCCT-AATACGACTCACTATAGGGC-3') and the ST6Gal exon I anti-sense primer md11 (5'-CTGCTTCTGGCTAATCTTCT-GGGGTTGG-3'). PCR amplification of ST6Gal sequence from contaminating genomic DNA is not possible because ST6Gal gene does not contain sequences that will specifically anneal the anchor primer. Touchdown PCR parameters are 94°C for 1 min, 5 cycles of 94°C for 30 s, 72°C for 4 min, 5 cycles of 94°C for 30 s, 70°C for 4 min, and finally 25 cycles of 94°C for 20 s, 68°C for 4 min. The PCR products were cloned into the plasmid vector pCR2.1 (Invitrogen) and sequenced.

For primer extention/5'-RACE analysis shown in Figure 2, primers mST1-LP-1 or mST1-LP-2 were used instead of mST1-P1 for reverse transcription. PCR amplification was achieved using the anchor primer AP1 and either mST1-LP-1 or mST1-LP-2. The sequences for mST1-LP-1 and mST1-LP-2 are denoted in Figure 2. 5'-RACE products were routinely cloned into pCR2.1. Clones were selected at random and sequence analyzed. For sialyltransferase enzymatic assays, the activity of total cell homogenates toward asialotransferrin was determined in the range of linearity with respect to protein concentration, determined according to the Lowry method as previously described (Dall'Olio *et al.*, 1996).

Acknowledgments

Abbreviations

LM, lactating mouse gland; NMG, normal mouse mammary gland; PCR, polymerase chain reaction; RACE, rapid amplication of CDNA ends; SHI, Shionogi; Sia, sialic acid.

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